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(54) Title: MATRIX METALLOPROTEINASE PROENZYME ACTIVATOR

(57) Abstract

An MMP-proenzyme activator (MMP-PA) that is expressed by articular cartilage. An MP-PA, biologically active fragments and mutants thereof, the cleavage products generated by the enzyme, and compounds (e.g., antibodies or small molecules) that modulate the MMP-PA's activity can be used to diagnose, monitor, and treat conditions in which articular cartilage is either lost or overproduced.

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MATRIX METALLOPROTEINASE PROENZYME ACTIVATOR

Statement as to Federally Sponsored Research

This invention was made with National Institutes of Health grant RO-1 AR16265. The government may, therefore, have certain rights in the invention.

Background of the Invention

Osteoarthritis is a common and debilitating disease that primarily afflicts the elderly. Its most prominent feature is the loss of articular cartilage, the weight-bearing, wear-resistant tissue that provides near frictionless articulation within healthy synovial joints. Proteases secreted by the cartilage are believed to contribute to the loss of extracellular matrix in osteoarthritis. Many of these proteases belong to the matrix metalloproteinase (MMP) family.

Most MMPs are secreted as inactive zymogens, requiring proteolytic release of a conserved propeptide domain for activation. Increased levels of one such protease, MMP-3, has been detected in cartilage or cartilage explant culture medium obtained from patients who have osteoarthritis. MMP-3 is also known as active neutral metalloproteinase, stromelysin, proteoglycanase, and collagenase activator protein.

Summary of the Invention

The invention is based on the isolation of a MMP-proenzyme activator (MMP-PA) that is expressed by articular cartilage (additional characteristics of this novel polypeptide are described below). This enzyme, biologically active fragments and mutants thereof, the cleavage products generated by the enzyme, and compounds (*e.g.*, antibodies or small molecules) that modulate the enzyme's activity can be used to diagnose, monitor, and

treat conditions in which articular cartilage is either lost or overproduced.

Accordingly, the invention features an isolated MMP-PA polypeptide that: (a) activates a matrix metalloproteinase proenzyme (MMP); (b) is produced by articular cartilage; (c) lacks caseinase and gelatinase activity; (d) does not degrade bovine serum albumin (BSA) or plasminogen; and (e) is inhibited by 4-(2-aminoethyl)-benzenesulfonylfluoride (AEBSF) or benzamidine. The polypeptide is approximately 60-70 kDa in size, is stable at 55°C, and inactivated at 70°C.

The polypeptide can activate a number of MMPs, including MMP-3 and MMP-9 (which is also known as 92-k gelatinase). An MMP-PA of the invention activates a MMP by initiating the removal of some or all of the propeptide domain of the MMP. Activation can be observed and quantitated by assays, such as those described herein (*see, e.g.*, the Examples below and Figs. 2A and 2B), in which the activity of an MMP is recorded following incubation with a particular concentration of MMP-PA for a particular time, or recorded over time as a percentage of the activity obtained following activation of a MMP by APMA. After incubation of the MMP zymogen with MMP-PA, a marked increase in the cleavage of labeled casein is evident. Thus, MMP-PAs activate a metalloproteinase (*e.g.*, a MMP) if, following incubation with the metalloproteinase, the MMP-PA increases the ability of the metalloproteinase to cleave casein (or an appropriate substrate (which would be known to one of skill in the art, *e.g.*, gelatin would be an appropriate substrate in the event the MMP is MMP-2 or MMP-9)) by at least about 10%, *e.g.*, by 25%, 50%, 100%, or more (relative to the ability of the inactivated metalloproteinase (*i.e.*, metalloproteinase untreated with MMP-PA) to cleave casein).

An MMP-PA of the invention lacks caseinase and gelatinase activity (when analyzed, *e.g.*, on substrate gels prepared and run essentially as described by Heussen *et al.* (*Anal. Biochem.* 102:196-202, 1980; see also the "Methods" section of the Examples and Towle *et al.*, *Biochem. Biophys. Res. Commun.* 247:324-331, 1998).

An MMP-PA of the invention can lack the ability to degrade bovine serum albumin (BSA) or plasminogen. The electrophoretic mobilities of BSA and plasminogen can be assessed, *e.g.*, after incubation under conditions that completely convert proMMP-3 to an active form (as described further below). Degradation (or digestion) of BSA and/or plasminogen can be assessed by SDS gel electrophoresis (as in the Examples, below; see also Towle *et al.*, *Biochem. Biophys. Res. Commun.* 247:324-331, 1998). BSA and/or plasminogen will be at least 50%, more preferably 75%, and most preferably essentially 100% intact (*i.e.*, undegraded) following incubation with an MMP-PA of the invention under the conditions described below (*i.e.*, incubation in 15 μl B-50 with 22 ng of a MMP-PA for 18 hours at 37°C).

An MMP-PA of the invention is stable at 55°C and inactive at 70°C. That is, preincubating the MMP-PA at 55°C for 20 minutes decreases subsequent MMP activation less than 90% (relative to the same MMP-PA preincubated at 37°C for 20 minutes. Similarly, preincubating the MMP-PA at 70°C for 20 minutes decreases subsequent MMP activation more than 50% (e.g., 70% 80% or even more than 90%).

An MMP-PA of the invention can be inhibited by 4-(2-aminoethyl)-benzenesulfonylfluoride (AEBSF) or benzamidine. To assess inhibition, an MMP can be incubated with an MMP-PA and any given additive (which may inhibit, stimulate, or have no effect on the MMP-PA's activity). Active MMP can then be measured using [^{14}C]-

casein as a substrate (for more specific parameters useful in carrying out such an assay, see the Examples below and Table 3). While proMMP-3 activation by MMP-PA is inhibited by 4-(2-aminoethyl)-benzenesulfonyl-fluoride (AEBSF) and benzamidine, the enzyme was not effectively inhibited by any other serine proteinase inhibitor tested (*see* Table 3).

The MMP-PA of the invention can be administered to an individual to treat a condition characterized by undesirable growth of tissue (*e.g.*, undesirable bone or cartilage formation as occurs, for example, in benign tumors). Alternatively, compounds that inhibit MMP-PA expression or activity can be identified and administered to an individual to treat a condition characterized by undesirable bone or cartilage degradation (*e.g.*, an arthritic condition such as osteoarthritis or rheumatoid arthritis). Inhibiting MMP-PA activity can also be useful in treating a patient who has a tumor, as preventing degradation of the extracellular matrix (ECM) can prevent tumor cells from migrating to other sites (*i.e.*, metastasis).

In another embodiment, the invention features antibodies that specifically bind MMP-PAs. As described further below, these antibodies can be monoclonal or polyclonal antibodies and can inhibit MMP activation by MMP-PAs. Inhibition of enzyme activity can be assessed in numerous ways, which will be readily apparent to one of ordinary skill in the art. For example, one can assess MMP-PA activity directly (*e.g.*, by assessing the ability of an MMP-PA to initiate cleavage of a MMP, *e.g.*, MMP-3 or MMP-9). Alternatively, one can assess MMP-PA activity indirectly, *i.e.*, by assessing the activity of an MMP upon which it acts. For example, one can assess MMP-PAs by assessing the ability of MMP-3 to cleave casein. An MMP-PA of the invention (*e.g.*, a fragment or

other mutant of the full length MMP-PA described herein) will initiate cleavage of an MMP (*e.g.*, MMP-3 or MMP-9) so that that MMP degrades casein at least about 10%, *e.g.*, 25%, 50%, or 100% more than it did prior to activation by the MMP-PA. Also within the invention are antibodies (*e.g.*, monoclonal antibodies) that recognize MMP neoepitopes.

The substances described herein, including agents that stimulate or inhibit MMP-PA expression or activity (*e.g.*, anti-MMP-PA antibodies) and MMP-PA polypeptides, or biologically active portions thereof (which can be identified by any assay, such as those described herein, for assessing the activity of the full length MMP-PA), can be incorporated into pharmaceutical compositions that, optionally, include pharmaceutically acceptable carriers.

Another aspect of the invention provides methods for detecting MMP-PA expression or activity in a biological sample (*e.g.*, a sample of blood, cartilage, or synovial fluid). The method can be carried out, for example, by contacting the biological sample with a nucleic acid probe that hybridizes with MMP-PA MRNA, an anti-MMP-PA antibody, or an agent that, directly or indirectly, detects MMP-PA activity (*e.g.*, an agent that detects activation of MMP-3 or MMP-9, *e.g.*, by detecting the products that are formed when one of these MMPs is converted from an inactive to an active form). Biological samples include samples of tissues, cells, or fluids isolated from a subject, as well as tissues, cells, or fluids present within a subject. Thus, the detection methods of the invention (whether employed to diagnose a patient, treat a patient, monitor a clinical trial, etc.) can be used to analyze a biological sample *in vitro* or *in vivo*. Preferably, the biological sample

is a sample of cartilage, synovial fluid, or blood, which can be obtained from a subject by conventional means.

Thus, methods in which MMP-PAs, or the products formed when these proteinases are active, are detected can be practiced to make or confirm a medical diagnosis (*e.g.*, that a patient has osteoarthritis or any other condition in which articular cartilage is lost) or to monitor the progression of articular cartilage loss (*e.g.*, during a clinical trial or treatment regime). For example, in a clinical trial of an agent that is designed to prevent, slow, or halt the loss of articular cartilage (by, *e.g.*, inhibiting an MMP-PA of the invention), the expression or activity of the MMP-PA can be assessed in groups of treated and untreated patients and correlated with the loss of articular cartilage.

Another aspect of the invention features a method for modulating MMP-PA activity. The method can be carried out, for example, by contacting a cell with an agent that modulates (*i.e.*, inhibits or stimulates) MMP-PA expression or activity. Preferably, MMP-PA expression or activity is modulated *in vivo*. The modulatory agent can be an antibody that specifically binds to MMP-PA or an agent that modulates transcription of a MMP-PA gene, splicing of a MMP-PA mRNA, or translation of a MMP-PA mRNA.

In another aspect, the invention provides a method for identifying a compound that binds to or modulates the activity of a MMP-PA protein. In general, such methods entail measuring a biological activity of MMP-PA in the presence and absence of a test compound and identifying those compounds which alter the activity of MMP-PA.

Similarly, the invention features methods for identifying a compound that modulates the expression of MMP-PA by measuring the expression of MMP-PA in the

presence and absence of a compound. A change in expression in the presence of the compound, relative to that seen in the absence of the compound, indicates that the compound modulates the expression of MMP-PA.

In one embodiment, the methods of the present invention are used to treat a subject having a disorder characterized by aberrant MMP-PA activity (*e.g.*, an arthritic condition, such as osteoarthritis) by administering an agent that is a MMP-PA modulator to the subject. Where the patient requires stimulation of MMP-PA activity, the MMP-PA modulator can be a MMP-PA protein. The MMP-PA modulator can also be a peptide, peptidomimetic, or other small molecule.

Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Brief Description of the Drawings

Fig. 1A is a gel filtration chromatograph (Sephacryl S-200) of MMP-PA. Active material (400 μg) obtained from the Matrex Gel Green step was chromatographed on a Sephacryl S-200 column equilibrated in B-50. Two ml fractions were collected and monitored for protein (estimated from absorbance at 280 nM). Aliquots of column fractions (0.5 μl of 2 ml) were tested for the capacity to activate bovine proMMP-3 using [^{14}C]-casein as a substrate for active MMP-3. Fig. 1B is a photograph of an SDS-polyacrylamide gel. Proteins in

fractions (20 μl of 2 ml) corresponding to the major peak of activity (Fig. 1A) were analyzed by SDS-PAGE under reducing conditions. Protein standards (bovine serum albumin, ovalbumin and carbonic anhydrase; Rainbow protein standards, Amersham) are indicated by bars at left. Fig. 1C is a photograph of an SDS-polyacrylamide gel. Aliquots (5 μl) of each fraction shown in Fig. 1B were tested for the capacity to cleave 300 ng bovine proMMP-3 in the presence of excess TIMP-1, with digestion products resolved on an SDS polyacrylamide gel. The proenzyme form of MMP-3 is indicated by arrows. The gels were stained with silver.

Figs. 2A and 2B are line graphs indicating activation of human fibroblast proMMP-3 by MMP-PA. In Fig. 2A, human proMMP-3 (37.6 μg/ml) was incubated for 4 hours with various amounts of MMP-PA in 10 μl B-50. Subsequent to the activating incubation, active MMP-3 in 3 μl of each reaction product was measured using labeled casein as a substrate. Data are expressed as a percentage of the casein degraded by APMA-activated MMP-3. Casein digestion by MMP-PA (2.2 μg/ml for 4 hours) was undetectable in this assay. In Fig. 2B, human proMMP-3 (33 μg/ml) was incubated for various times without exogenous activator (open circles) or with 1.1 μg/ml MMP-PA (filled circles) in 10 μl B-50. Active MMP-3 in 2 μl of each reaction product was measured using the labeled casein substrate, with activity expressed as a percentage of that obtained following activation by APMA.

Fig. 3 is a photograph of an SDS-polyacrylamide gel illustrating bovine proMMP-3 activation intermediates generated by MMP-PA. Bovine proMMP-3 (1 μg) was incubated in 20 μl B-50 without exogenous activator (lane 2), with 1 mM APMA (lane 3), or with various amounts of MMP-PA (11 ng, 22 ng, or 44 ng, lanes 4-6) in the presence of human recombinant TIMP-1. Reaction products

were resolved by SDS-PAGE, and the gels were stained with Coomassie Blue R250 Lane 1 contains proMMP-3 without incubation, and arrows indicate the MMP-3 activation products that accumulated during incubation with MMP-PA.

Fig. 4 is a diagrammatic representation of proMMP-3, which includes a propeptide (SEQ ID NO:__), a central catalytic domain, and a C-terminal hemopexin domain. Removal of some or all of the propeptide domain activates the enzyme. MMP-PA is predicted to hydrolyze the methionine$_{70}$-arginine$_{71}$ and arginine$_{71}$-lysine$_{72}$ peptide bonds in the human MMP-3 propeptide based on the alignment of sequences of bovine MMP-3 activation intermediates generated by this enzyme. At least one larger Mr activation intermediate was transiently present during incubation of proMMP-3 with MMP-PA, but it did not accumulate to levels sufficient for sequence analysis. The boxed region in the human MMP-3 propeptide is susceptible to a variety of proteinases, and subsequent MMP-3-dependent cleavage at the site indicated by the arrow generates a unique active species with a phenylalanine amino terminus.

Detailed Description

The present invention is based on the isolation of an activator of metalloproteinases (*e.g.*, matrix metalloproteinases).

I. Isolated MMP-PA Proteins and Anti-MMP-PA Antibodies

Isolated MMP-PAs, biologically active portions thereof, and polypeptide fragments can be used as immunogens to generate anti-MMP-PA antibodies. Native MMP-PAs can be isolated from cells or tissues by an appropriate purification scheme using standard protein purification techniques. MMP-PAs can also be recombinantly produced or chemically synthesized using

techniques routinely practiced by those of ordinary skill in the art.

As used herein, the term "polypeptide" refers to a chain of at least two amino acid residues, which may or may not be modified (*e.g.*, glycosylated). The terms "polypeptide," "peptide," and "protein" are considered equivalent and may be used interchangeably herein. An "isolated" or "purified" polypeptide is substantially free of contaminating proteins or other cellular materials associated with the cell or tissue from which the polypeptide (*e.g.*, an MMP-PA) was isolated (or in which the polypeptide was recombinantly produced) or from chemical precursors or other substances used in the process of chemically synthesizing the polypeptide. Preparations of MMP-PAs that are "isolated" or "purified" are preparations of MMP-PAs that have less than about 30%, 20%, 10%, or 5% (by dry weight) of non-MMP-PA protein (also referred to herein as a "contaminating protein"). When an MMP-PA, or a fragment (*e.g.*, a biologically active portion) thereof is recombinantly produced, it is preferably substantially free of culture medium, *i.e.*, culture medium represents less than about 30%, 20%, 10%, or 5% of the volume of the protein preparation.

As stated above, an isolated MMP-PA, or a portion or fragment thereof, can be used as an immunogen to generate antibodies that bind MMP-PA. These antibodies can be generated using standard techniques for preparing polyclonal and monoclonal antibodies.

More specifically, MMP-PA immunogens are typically used to prepare antibodies by immunizing a suitable subject (*e.g.*, a rabbit, goat, mouse, or other mammal) with the immunogen. An appropriate immunogenic preparation can contain, for example, purified MMP-PAs, recombinantly expressed MMP-PAs, or chemically

synthesized MMP-PAs. The preparation can also include an adjuvant, such as Freund's complete or incomplete adjuvant, or a similar immunostimulatory agent. Immunization of a suitable subject with an immunogenic MMP-PA preparation induces a polyclonal anti-MMP-PA antibody response.

The term "antibody" as used herein refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, *i.e.*, molecules that contain an antigen binding site that specifically binds an antigen (*e.g.*, an MMP-PA). A molecule that specifically binds to an MMP-PA is a molecule that binds an MMP-PA, but does not substantially bind other molecules in a sample, for example, a biological sample, which contains an MMP-PA. Examples of immunologically active portions of immunoglobulin molecules include F(ab) and $F(ab')_2$ fragments which can be generated by treating an antibody with an enzyme such as pepsin. The invention provides polyclonal and monoclonal antibodies that bind an MMP-PA. The terms "monoclonal antibody" and "monoclonal antibody composition," as used herein, refer to a population of antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of MMP-PA. A monoclonal antibody composition thus typically displays a single binding affinity for a particular MMP-PA with which it immunoreacts.

Antibodies that specifically bind to MMP neoepitopes (newly created epitopes having new amino and carboxy terminal ends, which are generated when a MMP is cleaved) are also within the scope of the invention. Antibodies raised against synthetic peptides corresponding to these epitopes can be used to identify or detect the cleaved MMP products in a biological fluid. More specifically, antibodies can be generated against

the following peptides: KDVKQFV (SEQ ID NO:1), RRKDSGP (SEQ ID NO:2), SDTLEVM (SEQ ID NO:3), RKPRCGV (SEQ ID NO:4), GYTRVAEM (SEQ ID NO:5), RGESKSL (SEQ ID NO:6), SATLKAM (SEQ ID NO:7), and RTPRCGV (SEQ ID NO:8). Any of these polypeptide immunogens (as well as others disclosed herein) can be linked to keyhole limpet hemocyanin.

Polyclonal anti-MMP-PA antibodies can be prepared as described above by immunizing a suitable subject with a MMP-PA immunogen. The anti-MMP-PA antibody titer in the immunized subject can be monitored over time by standard techniques, such as with an enzyme linked immunosorbent assay (ELISA) using immobilized MMP-PA. If desired, the antibody molecules directed against MMP-PA can be isolated from the mammal (*e.g.*, from the blood) and further purified by well-known techniques, such as protein A chromatography to obtain the IgG fraction. At an appropriate time after immunization, for example, when the anti-MMP-PA antibody titers are highest, antibody-producing cells can be obtained from the subject and used to prepare monoclonal antibodies by standard techniques, such as the hybridoma technique originally described by Kohler and Milstein (*Nature* 256:495-497, 1975), the human B cell hybridoma technique (Kozbor *et al.*, *Immunol. Today* 4:72, 1983), the EBV-hybridoma technique (Cole *et al.*, *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96, 1985) or trioma techniques. The technology for producing various monoclonal antibody hybridomas is well known (see, *e.g.*, Coligan *et al.* (eds.) *Current Protocols in Immunology* John Wiley & Sons, Inc., New York, NY, 1994). Briefly, an immortal cell line (typically a myeloma) is fused to lymphocytes (typically splenocytes) from a mammal immunized with a MMP-PA immunogen as described above, and the culture supernatants of the resulting hybridoma cells are

screened to identify a hybridoma producing a monoclonal antibody that binds MMP-PA.

Any of the many well known protocols used for fusing lymphocytes and immortalized cell lines can be applied for the purpose of generating an anti-MMP-PA monoclonal antibody (see, *e.g.*, Current Protocols in Immunology, *supra*; Galfre et *al.*, *Nature* 266:55052, 1977; R.H. Kenneth, in *Monoclonal Antibodies: A New Dimension In Biological Analyses*, Plenum Publishing Corp., New York, NY, 1980; and Lerner *Yale J. Biol. Med.* 54:387-402, 1981). Moreover, one of ordinary skill in the art will appreciate that there are many variations of such methods which would also be useful in the context of the present invention. Typically, the immortal cell line (*e.g.*, a myeloma cell line) is derived from the same mammalian species as the lymphocytes. For example, murine hybridomas can be made by fusing lymphocytes from a mouse immunized with an immunogenic preparation of MMP-PA with an immortalized mouse cell line, for example, a myeloma cell line that is sensitive to culture medium containing hypoxanthine, aminopterin, and thymidine ("HAT medium"). Any of a number of myeloma cell lines can be used as a fusion partner according to standard techniques, for example, the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14 myeloma lines. These myeloma lines are available from the American Type Culture Collection (Manassas, VA). Typically, HAT-sensitive mouse myeloma cells are fused to mouse splenocytes using polyethylene glycol (PEG). Hybridoma cells resulting from the fusion are then selected using HAT medium, which kills unfused and unproductively fused myeloma cells (unfused splenocytes die after several days because they are not transformed). Hybridoma cells producing a monoclonal antibody of the invention are detected by screening the hybridoma culture

supernatants for antibodies that bind MMP-PA, for example, using a standard ELISA assay.

Instead of preparing monoclonal antibody-secreting hybridomas, a monoclonal anti-MMP-PA antibody can be identified and isolated by screening a recombinant combinatorial immunoglobulin library (*e.g.*, an antibody phage display library) with MMP-PA to thereby isolate members of the immunoglobulin library that bind MMP-PA. Kits for generating and screening phage display libraries are commercially available (*e.g.*, the Pharmacia *Recombinant Phage Antibody System*, Catalog No. 27-9400-01; and the Stratagene *SurfZAP™ Phage Display Kit*, Catalog No. 240612).

An anti-MMP-PA antibody (*e.g.*, a monoclonal antibody) can be used to isolate MMP-PA by standard techniques, such as affinity chromatography or immunoprecipitation. An anti-MMP-PA antibody can facilitate the purification of natural MMP-PA from cells and of recombinantly produced MMP-PA from host cells. Moreover, an anti-MMP-PA antibody can be used to detect MMP-PA protein (*e.g.*, in a cellular lysate or cell supernatant) in order to evaluate the abundance and pattern of expression of the MMP-PA protein. Anti-MMP-PA antibodies can be used diagnostically to monitor protein levels in tissue as part of a clinical testing procedure to, for example, determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling the antibody to a detectable substance. Detectable substances are well known to those of ordinary skill in the art and include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials.

II. Pharmaceutical Compositions

The MMP-PA polypeptides and anti-MMP-PA antibodies (also referred to herein as "active compounds") of the invention can be incorporated into pharmaceutical compositions suitable for administration to a subject (*e.g.*, a human patient). These compositions typically include the polypeptide or antibody and a pharmaceutically acceptable carrier. As used herein, the term "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Use of these carriers in therapeutic compositions is contemplated, except insofar as they are incompatible with the active compound (a determination one of ordinary skill in the art can readily make). Supplementary active compounds can also be incorporated into the compositions.

Naturally, a pharmaceutical composition of the invention will be formulated in a manner compatible with its intended route of administration. Examples of routes of administration include oral administration and parenteral administration. For example, a pharmaceutical composition can be formulated for oral, intravenous (or intraarterial), intradermal, intramuscular, subcutaneous, oral, transdermal, or transmucosal (*e.g.*, nasal or rectal) administration. Similarly, a pharamaceutical composition can be formulated for application to the area surrounding a joint (*e.g.*, injection into the synovial fluid). The solutions, suspensions, gels, ointments, or creams used for administration can include one or more of the following components: a sterile diluent such as water, saline solution, fixed oils, polyethylene glycols,

glycerine, propylene glycol, or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediamine-tetraacetic acid; buffers such as acetates, citrates, or phosphates; and agents for the adjustment of tonicity such as sodium chloride or dextrose. The pH of a pharmaceutical preparation can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. Moreover, the preparations can be enclosed in ampoules, disposable syringes, or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injection include sterile aqueous solutions (where the active ingredient is water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF; Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example,

parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, and sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound (*e.g.*, MMP-PAs or anti-MMP-PA antibodies) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, and sterilizing (*e.g.*, filter sterilizing) the solution. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and any other required ingredients (*e.g.*, ingredients selected from those enumerated above).

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches, and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an

excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring. Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams by methods generally known in the art.

In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those of ordinary skill in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those of ordinary skill in the art, for example, as described

in U.S. Patent No. 4,522,811. It is especially advantageous to formulate oral or parenteral compositions in the form of dosage units, as this provides for ease of administration and uniformity of dosage. A "dosage unit" refers to a physically discrete unit suitable as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound, the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

III. Uses and Methods of the Invention

The polypeptides, including antibodies, described herein can be used in one or more ways. For example, they can be used in: (a) screening assays, (b) predictive medicine (*e.g.*, in diagnostic assays, prognostic assays, monitoring clinical trials, and pharmacogenomics); and (c) methods of treatment (*e.g.*, in therapeutic and prophylactic regimes). MMP-PAs can interact with other cellular proteins and can be used to regulate MMP activity, which, in turn, regulates the degradation of extracellular matrices (*e.g.*, bone or cartilage). In addition, MMP-PAs can be used to screen drugs or compounds that modulate MMP-PA expression or activity, to treat disorders characterized by insufficient or excessive production of MMP-PAs, and to treat disorders characterized by production of MMP-PAs that have decreased or aberrant activity compared to the wild type MMP-PA (*e.g.*, mutant MMP-PAs). In addition,

anti-MMP-PA antibodies can be used to detect and isolate MMP-PAs and to modulate their activity.

The invention also encompasses novel agents identified by the screening assays described above, and uses for those agents (*e.g.*, in diagnostic and treatment regimes), as described herein.

Screening Assays

By practicing a method of the invention (*i.e.*, a "screening assay") one of ordinary skill in the art can identify modulators, *i.e.*, candidate or test compounds or agents (*e.g.*, peptides, peptidomimetics, small molecules, or other drugs) that bind to MMP-PAs or have a stimulatory or inhibitory effect on, for example, MMP-PA expression or MMP-PA activity.

For example, one can screen candidate or test compounds that bind to or otherwise modulate the activity of a MMP-PA polypeptide or a biologically active portion thereof. The test compounds can be obtained using any of the numerous libraries known in the art of combinatorial library methods, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the "one-bead one-compound" library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, *Anticancer Drug Des.* 12:145, 1997).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt *et al.*, *Proc. Natl. Acad. Sci. USA* 90:6909, 1993; Erb *et al. Proc. Natl. Acad. Sci. USA* 91:11422, 1994; Zuckermann *et al.*, *J. Med. Chem.* 37:2678, 1994; Cho

et al., *Science* 261:1303, 1993; Carrell *et al.*, *Angew. Chem. Int. Ed. Engl.* 33:2059, 1994; Carell *et al.*, *Angew. Chem. Int. Ed. Engl.* 33:2061, 1994; and Gallop *et al.*, *J. Med. Chem.* 37:1233, 1994.

Libraries of compounds may be presented in solution (*e.g.*, Houghten, *Bio/Techniques* 13:412-421, 1992), or on beads (Lam, *Nature* 354:82-84, 1991), chips (Fodor, *Nature* 364:555-556, 1993), bacteria (U.S. Patent No. 5,223,409), spores (U.S. Patent Nos. 5,571,698; 5,403,484; and 5,223,409), plasmids (Cull *et al.*, *Proc. Natl. Acad. Sci. USA* 89:1865-1869, 1992) or on phage (Scott and Smith, *Science* 249:386-390, 1990; Devlin, *Science* 249:404-406, 1990; Cwirla *et al.*, *Proc. Natl. Acad. Sci. USA* 87:6378-6382, 1990; and Felici, *J. Mol. Biol.* 222:301-310, 1991).

An assay to screen candidate or test compounds that bind to or otherwise modulate the activity of a MMP-PA polypeptide can be a cell-based assay in which a cell that expresses a MMP-PA, or a biologically active portion thereof, is contacted with a test compound and the ability of the test compound to bind to a MMP-PA protein determined. The cell, for example, can be a yeast cell or a cell of mammalian origin. Determining the ability of the test compound to bind to the MMP-PA protein can be accomplished, for example, by coupling the test compound to a radioisotope or enzymatic label such that binding of the test compound to the MMP-PA, or biologically active portion thereof, can be determined by detecting the labeled compound in a complex. For example, test compounds can be labeled with ^{125}I, ^{35}S, ^{14}C, or ^{3}H, either directly or indirectly, and the radioisotope can be detected by direct counting of radioemmission or by scintillation counting. Alternatively, test compounds can be enzymatically labeled with, for example, horseradish peroxidase, alkaline phosphatase, or

luciferase, and the enzymatic label detected by determination of conversion of an appropriate substrate to product. The assay can be carried out, for example, by contacting a cell that expresses an MMP-PA, or a biologically active portion thereof, with a compound known to bind MMP-PA to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to interact with a MMP-PA protein. One can determine whether the test compound has the ability to interact with a MMP-PA protein by, for example, determining whether (and optionally, to what extent) the test compound has the ability to preferentially bind to the MMP-PA, or a biologically active portion thereof, in a manner comparable to that of the known compound.

Alternatively, a cell-based assay can be carried out by contacting a cell expressing a MMP-PA, or a biologically active portion thereof, with a test compound and determining whether (and, optionally, to what extent) the test compound modulates (*e.g.*, stimulates or inhibits) the activity of the MMP-PA protein (or the biologically active portion thereof). Determining whether the test compound modulates the activity of MMP-PA, or a biologically active portion thereof, can be accomplished, for example, by determining whether the MMP-PA binds to or otherwise interacts with a MMP-PA target molecule (*i.e.*, a molecule with which a MMP-PA binds or interacts in nature, for example, a MMP-PA substrate (*e.g.*, MMP-3 or MMP-9), a molecule in the extracellular milieu, or a molecule associated with the external surface of a cell membrane. A MMP-PA target molecule can be a non-MMP-PA molecule or a MMP-PA the present invention.

Determining whether the MMP-PA binds to or interacts with a MMP-PA target molecule can be

accomplished by any one of the methods described above for determining direct binding. For example, determining the ability of an MMP-PA to bind to or otherwise interact with a MMP-PA target molecule can be accomplished by determining the activity of the target molecule. For example, the activity of the target molecule can be determined by detecting MMP proprotein activation or proteolysis (*e.g.*, in the assay presented in the examples below).

A cell-free assay can also be carried out by contacting a MMP-PA, or biologically active portion thereof, with a test compound and determining whether (and optionally, to what extent) the test compound binds to the MMP-PA, or the biologically active portion thereof. Binding between the test compound and the MMP-PA can be determined either directly or indirectly, as described above. The assay can include contacting the MMP-PA, or a biologically active portion thereof, with a known compound that binds MMP-PA to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to interact with a MMP-PA protein. Thus, determining the ability of the test compound to interact with a MMP-PA includes determining the ability of the test compound to preferentially bind to MMP-PA, or a biologically active portion thereof, as compared to the known compound.

A cell-free assay can also be carried out by contacting a MMP-PA, or a biologically active portion thereof, with a test compound and determining the ability of the test compound to modulate (*e.g.*, stimulate or inhibit) the activity of the MMP-PA protein, or a biologically active portion thereof. Determining the ability of the test compound to modulate the activity of MMP-PA can be accomplished, for example, by determining

the ability of the MMP-PA protein to bind to a MMP-PA target molecule by one of the methods described above for determining direct binding. Alternatively, determining the ability of the test compound to modulate the activity of MMP-PA can be accomplished by determining the ability of the MMP-PA to further modulate a MMP-PA target molecule. For example, one can determine the catalytic or enzymatic activity of the target molecule on an appropriate substrate, as previously described.

A cell-free assay can also be carried out by contacting the MMP-PA, or a biologically active portion thereof, with a known compound that binds MMP-PA to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to interact with a MMP-PA protein. Determining the ability of the test compound to interact with a MMP-PA protein can include determining the ability of the MMP-PA protein to preferentially bind to or modulate the activity of a MMP-PA target molecule, such as MMP-3 or MMP-9.

In any of the assays or methods described herein, it may be desirable to immobilize either the MMP-PA or its target molecule to facilitate separation of complexed from uncomplexed forms of one or both of the proteins, as well as to accommodate automation of the assay. Binding of a test compound to MMP-PA, or interaction of MMP-PA with a target molecule in the presence and absence of a candidate compound, can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtitre plates, test tubes, and micro-centrifuge tubes. The solid support used for immobilization can include an array, such as a microtitre plate, or beads which can be separated from a mixture. Following incubation, the microtitre plate wells are washed to remove any unbound components, and the complex

determined either directly or indirectly, for example, as described above. Alternatively, the complexes can be dissociated from the solid support, and the level of MMP-PA binding or activity determined using standard techniques.

Other techniques for immobilizing proteins on matrices can also be used in the present screening assays. For example, either MMP-PA or its target molecule can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated MMP-PA or target molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (*e.g.*, biotinylation kit, Pierce Chemicals, Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemicals, Rockford, IL). Alternatively, antibodies reactive with MMP-PA or target molecules but which do not interfere with binding of the MMP-PA protein to its target molecule can be derivatized to the wells of the plate, and unbound target or MMP-PA trapped in the wells by antibody conjugation. Methods for detecting such complexes include immunodetection of complexes using antibodies reactive with the MMP-PA or target molecule, as well as enzyme-linked assays, which rely on detecting an enzymatic activity associated with the MMP-PA or target molecule.

The invention encompasses the novel agents identified by the screening assays described above, and uses for those agents (*e.g.*, in diagnostic and treatment regimes), as described herein.

More specifically, one can use diagnostic assays to determine MMP-PA activity in a biological sample (*e.g.*, a sample of blood, serum, cells, or tissue) and, thereby, determine whether an individual has a disease or disorder associated with aberrant MMP-PA expression or activity.

Similarly, one can use assays to determine whether an individual is at risk of developing a disease or disorder associated with aberrant MMP-PA activity. These assays can be used to identify an individual prophylactically, *i.e.*, prior to the symptomatic or overt onset of a disease or disorder characterized by or associated with aberrant MMP-PA activity.

The compositions and methods described herein can be used to monitor the influence of agents (*i.e.*, chemical agents or other compounds) on the activity of MMP-PA. For example, in a clinical trial of a pharmaceutical agent designed to treat or prevent an arthritic condition (*e.g.*, osteoarthritis) by inhibiting the activity of MMP-PA, one could determine the activity of MMP-PA in biological samples (*e.g.*, samples of synovial fluid) that were obtained from individuals participating in the clinical trial before and at least once during the trial. A reduction in MMP-PA activity in samples obtained after treatment was begun indicates that the agent administered provides an effective treatment for the arthritic condition. Similarly, a reduction in MMP-PA activity in samples obtained from patients being treated with the agent, relative to comparable samples obtained from untreated patients, indicates that the agent administered provides an effective treatment for the arthritic condition.

One can detect the presence or absence of MMP-PA (and, optionally, determine the level of MMP-PA expression or activity) in a biological sample by contacting the sample (*e.g.*, a sample obtained from a test subject) with a compound or an agent capable of binding to or otherwise interacting with MMP-PA in a way that reflects MMP-PA expression or activity.

One of the agents that can be used to detect MMP-PA is an antibody that specifically binds to MMP-PA. For

ease of detection, the antibody can be linked to a detectable marker. Antibodies can be polyclonal, or more preferably, monoclonal. The term "detectable marker," as used in the context of an antibody probe (or other type of probe for MMP-PA expression or activity), encompasses antibodies that are directly coupled (*i.e.*, physically linked) to a detectable substance, as well as antibodies that are indirectly linked to a detectable substance. For example, an indirect indicator of MMP-PA activity is a primary antibody that is detected by its ability to bind a fluorescently labeled secondary antibody.

In vitro techniques for detecting MMP-PA include enzyme linked immunosorbent assays (ELISAs), Western blot analyses, immunoprecipitations, and immunofluorescence. *In vivo* techniques for detecting MMP-PA include introducing a labeled anti-MMP-PA antibody into a subject. The antibody can be labeled, for example, with a radioactive marker whose presence and location in a subject can be detected by standard imaging techniques.

Those of ordinary skill in the art are accustomed to including "control" samples in experiments and would recognize that a biological sample from a control subject could be manipulated in parallel with biological samples from test subjects by, for example, contacting the control and test samples (*i.e.*, a biological sample obtained from a subject of interest (*e.g.*, a person who is thought to have a disease or disorder associated with aberrent MMP-PA expression or activity)) with a compound or agent capable of detecting MMP-PA. MMP-PA expression or activity in the control sample is then compared with the expression or activity of MMP-PA protein in the test sample.

Kits can be assembled for detecting MMP-PA expression or activity in a biological sample (a test and/or a control sample). These kits can be used, for

example, to determine if a subject is suffering from or is at increased risk of developing a disorder associated with aberrant MMP-PA expression or activity. A kit can include a labeled compound or agent that detects MMP-PA in a biological sample (*e.g.*, an anti-MMP-PA antibody) and one or more reagents for determining the amount of MMP-PA in the sample. Kits may also include instructions (*e.g.*, text or diagrams) useful in concluding that the tested subject is suffering from or is at risk of developing a disease or disorder associated with aberrant expression or activity of MMP-PA. For example, the instructions may disclose the normal range of a MMP-PA.

In the event a kit is an antibody-based kit, it may include: (1) a first antibody (*e.g.*, an antibody attached to a solid support) that specifically binds to MMP-PA; and, optionally, (2) a second antibody, which is different from the first antibody, and which specifically binds to MMP-PA or the first antibody and is conjugated to a detectable agent. Accordingly, the kit can also include components useful for detecting the detectable agent (*e.g.*, an enzyme or a substrate).

The kit can also include, for example, a buffering agent, a preservative, a protein stabilizing agent, a control sample, or a series of control samples. The control sample(s) can be assayed and compared to the test sample. The significance of such comparisons will be evident to those of ordinary skill in the art. Typically, each component of the kit will be enclosed within an individual container and all of the various containers will be contained within a single package along with instructions for determining whether the tested subject is suffering from or is at risk of developing a disease or disorder associated with aberrant expression or activity of MMP-PA.

Methods of Treatment

In one aspect, the invention provides a method for preventing in a subject, a disease or condition associated with an aberrant MMP-PA expression or activity, by administering to the subject an agent which modulates MMP-PA expression or at least one MMP-PA activity. Subjects at risk for a disease which is caused or contributed to by aberrant MMP-PA expression or activity can be identified by, a diagnostic assay as described herein. Administration of a prophylactic agent can occur prior to the manifestation of symptoms characteristic of the MMP-PA aberrancy, such that a disease or disorder is prevented or, alternatively, delayed in its progression. Depending on the type of MMP-PA aberrancy, for example, a MMP-PA agonist or MMP-PA antagonist agent can be used for treating the subject. The appropriate agent can be determined based on screening assays described herein.

Another aspect of the invention pertains to methods of modulating MMP-PA expression or activity for therapeutic purposes. The modulatory method of the invention involves contacting a cell with an agent that modulates one or more of the activities of MMP-PA. In one embodiment, the agent stimulates one or more of the biological activities of MMP-PA protein. Examples of such stimulatory agents include active MMP-PA protein. In another embodiment, the agent inhibits one or more of the biological activities of MMP-PA protein. Examples of such inhibitory agents include anti-MMP-PA antibodies. These modulatory methods can be performed *in vitro* (*e.g.*, by culturing the cell with the agent) or, alternatively, *in vivo* (*e.g.*, by administering the agent to a subject). As such, the present invention provides methods of treating an individual afflicted with a disease or disorder characterized by aberrant expression or activity

of a MMP-PA protein or nucleic acid molecule. In one embodiment, the method involves administering an agent (*e.g.*, an agent identified by a screening assay described herein), or combination of agents that modulates (*e.g.*, upregulates or downregulates) MMP-PA expression or activity. In another embodiment, the method involves administering a MMP-PA protein as therapy to compensate for reduced or aberrant MMP-PA expression or activity.

Stimulation of MMP-PA activity is desirable in situations in which MMP-PA is abnormally downregulated and/or in which increased MMP-PA activity is likely to have a beneficial effect. Conversely, inhibition of MMP-PA activity is desirable in situations in which MMP-PA is abnormally upregulated and/or in which decreased MMP-PA activity is likely to have a beneficial effect.

The invention is further illustrated by the following examples, which are provided to illustrate, not limit, the invention. The references, patents, and published patent applications cited throughout this application are hereby incorporated by reference in their entirety.

Examples

Methods

Materials. Bovine proMMP-3 was purified from the culture medium of IL-1-stimulated cartilage to a specific activity of 550 u/mg essentially as described in Treadwell *et al.* (*Arch. Biochem. Biophys.* 251:715-723, 1986). Human proMMP-3 purified from synovial fibroblast conditioned medium was purchased from Accurate Chemical and Scientific Corp. Link protein was purified from bovine articular cartilage by associative and dissociative density gradient centrifugation (Tang *et al.*, *J. Biol. Chem.* 254:10523-10531, 1979). Human recombinant IL-1 was a gift from Peter Lomedico, of

Hoffman LaRoche (Nutley, NJ), and human recombinant TIMP-1 was a gift from David Carmichael, of Synergen Corp. However, both these polypeptides are commercially available, for example, from CalBiochem (San Diego, CA).

Preparation of cartilage media. Articular cartilage was resected from the radial carpal joints of 7 to 11 day old calves. Cartilage from 4 joints (~40 gm) was washed with 50 volumes of sterile phosphate buffered saline (PBS) and placed in approximately 25 volumes of Dulbecco's modified Eagle's medium (DMEM) containing 15 mM HEPES (pH 7.4), penicillin (100 u/ml), streptomycin (50 μg/ml), and ascorbate (50 μg/ml). Cartilage was cultured in humidified air with 5% CO_2 at 37°C. After 24 hours the medium was discarded and replaced with fresh DMEM. Cultures were incubated at 37°C, and the media were harvested at 4 day intervals. Harvested media were adjusted to 0.02% NaN_3 and concentrated to dryness by the combined action of an Amicon Diaflow hollow fiber concentrator (3,000 molecular weight cutoff cartridge) followed by an Amicon concentrator fitted with a YM-10 membrane. Concentrated material was solubilized in 10 ml B-50 (20 mM tris HCl pH 7.8, 1 mM $CaCl_2$, 0.1 μM $ZnCl_2$, 0.02% NaN_3, 0.05 M NaCl) per liter cartilage conditioned medium and stored at -20°C until used for purification.

Protein analysis. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE) of protein samples was carried out according to the method of Laemmli (*Nature* 22:680-684, 1970). Gelatin degrading activity was analyzed on substrate gels prepared and run essentially as described by Heussen et *al.* (*Anal. Biochem.* 102:196-202, 1980; herein incorporated by reference in its entirety). Buffers used for purification contained 20 mM tris HCl (pH 7.8), 1 mM $CaCl_2$, 0.1 uM $ZnCl_2$, 0.02% NaN_3 with NaCl as indicated, for example, B125 has 125 mM NaCl in addition to the

components listed above. Protein concentrations were estimated from the absorbance at 280 nm, assuming 1 A_{280} unit is 1 mg/ml.

Assay procedures. At each purification step, fractionated material was tested for the ability to activate bovine proMMP-3 as follows. ProMMP-3 (100 ng) was incubated for 18 hours at 37°C with fractions to be tested in 10 µl B-50. Activation was then assessed using [^{14}C]-acetylated casein as a substrate for active MMP-3, with the amount of casein digested in 4 hours, using 15 µl, measured essentially as previously described in Treadwell *et al.* (*Arch. Biochem. Biophys.* 251:715-723, 1986). Appropriate controls were run in parallel to measure activity independent of added proMMP-3. Enzyme quantities at each stage of purification were adjusted to fall within the linear range of the assay. One unit of MMP-PA is defined as the amount of protein required to activate 50 ng of proMMP-3 in this assay. ProMMP-3 was activated by incubation with 1 mM APMA for 18 hours at 37°C to define full activation.

For the analysis of proteolytic processing of the MMP-3 zymogen, proMMP-3 was incubated with enzyme fraction to be tested for 18 hours at 37°C, and the extent of conversion from latent to lower molecular weight forms was estimated following resolution of reaction products by SDS-PAGE. Where indicated, a 4-fold molar excess of human recombinant TIMP-1 relative to MMP was included in the activating incubation mixture to inhibit MMP-dependent autocatalytic cleavage.

Purification of MMP-PA. Pooled concentrated cartilage conditioned medium (250 ml, approximately 1000 mg) was adjusted to 40% saturated NH_4SO_4, the pH was adjusted to 7.0, and the protein was precipitated on ice for 2 hours. Precipitated protein was removed by centrifugation (12,000 x g, for 30 min at 4°C), and the

supernatant was adjusted to 90% saturation with NH_4SO_4. Protein was allowed to precipitate overnight at 4°C, and the precipitates were recovered by centrifugation at 12,000 x g for 30 minutes at 4°C. Protein was dissolved in 10 ml B-125 and dialyzed (3,500 molecular weight cutoff) against the same buffer prior to application to DEAE Sephadex A50 resin equilibrated in B125 (50 ml resin). The column flow through (5 column volumes B-125) was concentrated by Amicon to apparent dryness. The concentrate was dissolved in 5 ml B-50 and applied to a 5 ml Matrex Gel Green A (Amicon Corp.) column equilibrated in B-50.

The Matrex Gel Green flow through was collected, concentrated to apparent dryness and dissolved in 1 ml B-50, and applied to a Sephacryl S-200 HR column (1.5 x 85 cm), equilibrated and run in B-50 at a flow rate of 1 ml/min. Fractions (2 ml) were monitored for MMP-PA by incubating 100 ng of proMMP-3 with 0.5 µl of each column fraction for 18 hours at 37°C and then measuring the amount of casein digested in 4 hours. Fractions from the Sephacryl S-200 HR column showing evidence of proMMP-3 activation in the initial screen were assayed at various dilutions as described above to determine units of activity. The fraction with the highest proMMP-3-activating activity was used for further characterization of MMP-PA.

Substrate specificity of MMP-PA. Various purified proteins (bovine serum albumin, link protein, plasminogen, and casein; 0.3 to 0.6 µg) were incubated in 15 µl B-50 with 22 ng MMP-PA for 18 hours at 37°C. For comparison, parallel incubations were carried out with protein alone or protein incubated with trypsin (22 ng). Reaction products were reduced and analyzed by SDS-PAGE.

NH_2-terminal sequence analysis of bovine proMMP-3 and link cleavage products generated during incubation with MMP-PA. Bovine proMMP-3 (8 μg) was incubated for 18 hours at 37°C with 275 ng of MMP-PA and 12.7 μg human rTIMP-1 in 150 μl B-50. Bovine link protein (8 μg) was incubated for 18 hours at 37°C with 165 ng MMP-PA in a 50 μl reaction. Digestion products were resolved by SDS-PAGE under reducing conditions and transferred to poly(vinylidene difluoride) membrane (PVDF) (Matsudaira, *J. Biol. Chem.* 262:10035-10038, 1987). After staining the transferred proteins with Coomassie Blue R-250, bands corresponding to the major proteolytic fragments were excised from the blot and sequenced using an Applied Biosystems Model 477A Protein Sequencer.

Sensitivity to thermal denaturation and inhibitors. MMP-PA (28 ng) was incubated at various temperatures for 20 minutes prior to testing its capacity to activate 300 ng bovine proMMP-3 in 18 hours at 37°C. The effect of proteinase inhibitors on proMMP-3 activation was similarly tested except that there was no preincubation step. Subsequent to the activating incubations, active MMP-3 was measured using [^{14}C]casein as a substrate in a 4 hour reaction.

The effect of chelating agents on MMP-PA proteolysis of bovine proMMP-3 and link protein was assessed by comparison of reaction products following SDS-PAGE. Recombinant TIMP-1 was included during the incubation to limit MMP-dependent proteolysis. To further explore the metal dependence of MMP-PA proteolytic activity, the enzyme buffer was first exchanged with 20 mM Tris HCl pH 7.8, 50 mM NaCl, 0.02% NaN_3 (TBS) by repeated dilution and concentration using a Nanosep centrifugal concentrator (10,000 molecular weight cutoff, Pall Corp.).

Table 1 summarizes the procedure used to isolate proMMP-3-activating activity from bovine articular cartilage conditioned medium. Twenty-five liters of explant culture medium (1000 A280 units) yielded 22 μg protein at the S-200 stage of purification with a 2500-fold increase in specific activity over the starting conditioned medium.

Table 1. Purification of MMP-PA

Step	Total protein	Total Activity	Yield	Specific Activity	Purification
	mg	units*	%	units/mg	-fold
Crude**	1,000	187,500	-	187	-
DEAE	10.1	163,000	87	16,300	87
Green	0.4	8,700	4.6	21,750	116
Matrex					
S-200	0.022	10,200	5.4	463,600	2,479

At each stage of purification, active material was analyzed by SDS-PAGE and zymography on casein and gelatin substrate gels in order to trace the removal of contaminants. The NH_4SO_4 fractionation removed the bulk of the proteoglycans, which otherwise interfered with subsequent purification steps, and some of the carboxy-terminal propeptide of type II collagen (the major protein in cartilage conditioned medium). Chromatography on DEAE-Sephadex removed most of the remaining proteoglycans and a 57 kDa casein degrading activity (presumably proMMP-3). The Matrex Gel Green resin removed most of the gelatinase activity (MMP-2 and MMP-9), the remaining C-propeptide, and many low abundance proteins.

In Table 1, one "unit" is the amount of protein required to activate 50 ng proMMP-3, as determined by digestion of [^{14}C]casein using the assay described above (see "Methods"), and the row labeled "crude" refers to total protein in conditioned medium. Activity at early stage cannot be accurately measured because of interference from other

components (other proteinases, substrates, and inhibitors).

The proMMP-3 activating activity eluted from Sephacryl S-200 with an apparent molecular weight of about 43,000 (Fig. 1A). The proteins in each fraction were resolved on SDS polyacrylamide gels under reducing conditions, and the protein bands were visualized by silver staining (Fig. 1B). Aliquots of Sephacryl S-200 column fractions spanning the peak of activity (fractions 34-41) were incubated with proMMP-3, and activation products were resolved on a SDS polyacrylamide gel (Fig. 1C). Visual inspection of the gel profiles revealed that the major peak of proMMP-3-activating activity (fractions 35-38) paralleled the intensity of bands corresponding to a "doublet" at about 72 kDa and a somewhat diffuse band at about 52 kDa. Active fractions from the S-200 column (5 μl) were tested for endogenous "caseinase" and "gelatinase" activity in the absence of added proMMP-3. Sephacryl S200 fraction number 37, lacking detectable gelatinase or caseinase activity, was used to further characterize MMP-PA.

Time and dose-dependent activation of proMMP-3 by MMP-PA. Human proMMP-3 was predominantly in the latent form, migrating as a 59/57 kDa doublet on SDS-PAGE. Some activation of latent enzyme occurred during incubation in the absence of exogenous activator, as indicated by the increased digestion of the [^{14}C]-labeled casein substrate with time of incubation (Fig. 2A). MMP-PA itself had no caseinase activity detectable by this assay. After incubation of the MMP-3 zymogen with MMP-PA, a marked increase in the cleavage of labeled casein was evident. Activation of proMMP-3 was evident after 1 hour incubation with 1 μg/ml MMP-PA (Fig. 2).

Stepwise processing of proMMP-3 by MMP-PA. In order to allow the accumulation of activation

intermediates generated by MMP-PA, activation reactions were carried out in the presence of TIMP-1. At least three species in the size range 45,000 to 53,000 daltons were detected when reaction products were resolved by SDS-PAGE (Fig. 3, lanes 4-6).

Substrate specificity of MMP-PA proteolysis. In order to further investigate the specificity of MMP-PA, various proteins were incubated with the enzyme prior to analysis of digestion products by SDS-PAGE. After incubation under conditions that completely converted proMMP-3 to an active form, the electrophoretic mobilities of bovine serum albumin and plasminogen were unchanged. However, cartilage link protein was cleaved to generate a major reaction product slightly smaller than the pristine molecule. By comparison, trypsin degraded all of these substrates to sub-18 kDa polypeptides that were electrophoresed from the gel.

Definition of MMP-PA cleavage sites in proMMP-3 and link protein. To define more clearly the substrate specificity of MMP-PA's proteolytic activity, the sequences of the amino termini of the major digestion products of proMMP-3 and link protein were determined (Table 2).

Table 2. NH_2-terminal sequences of digestion products of bovine proMMP-3 and link protein accumulating during incubation with MMP-PA.

	Bovine proMMP-3						Bovine Link Protein	
Cycle	Sequence 1*						Sequence 2	
1	R		K				R	
2	K		P				V	
3	P		R				I	
4	R		X				H	
5	X		G				I	
6	G		I				Q	
7	I		P				A	
8	P		X				E	

* The proMMP-3 digestion product gave a mixed sequence. The two sequences were resolved by comparison with the sequence for human proMMP-3.

This analysis demonstrated that bovine proMMP-3 was cleaved during incubation with MMP-PA at sites corresponding in the human homolog to the methionine$_{70}$-arginine$_{71}$ and arginine$_{71}$-lysine$_{72}$ bonds in the propeptide to create two 46,000 M_r products that appeared as a single band on the Coomassie Blue stained blot (Fig. 4). The N-terminal sequence of the unique link digestion product detected indicated that MMP-PA cleaved the aspartic acid$_{12}$-arginine$_{13}$ bond to generate about a 42 kDa catabolite.

Functional characterization. MMP-PA was stable to incubation at 55°C, partially inactivated at 60°C, and completely inactivated at 70°C.

Bovine proMMP-3 (300 ng) was incubated with 28 ng MMP-PA for 18 hours with each of the additives shown in Table 3. Active MMP-3 was measured using [^{14}C]-casein as a substrate. ProMMP-3 activation by MMP-PA was inhibited by 4-(2-aminoethyl)-benzenesulfonyl-fluoride (AEBSF) and benzamidine, but the enzyme was not effectively inhibited by any other serine proteinase inhibitor tested (*see* Table 3). The enzyme was not inhibited by agents that target members of the aspartic or cysteine class of proteinases (E-64, N-ethyl maleimide, pepstatin).

Table 3. Sensitivity of MMP-PA to inhibitors.

INHIBITOR	CONCENTRATION	INHIBITION (%)*
Benzamidine	0.8 mM	67
Benzamidine	1.6 mM	84
AEBSF	1 mM	100
PMSF	1 mM	25
DCIC	0.2 mM	0
TLCK	0.14 mM	0
aprotinin	0.3 μM	11
SBTI	5 μM	3
α1 PI	0.2 μM	4
leupeptin	1 μM	2
E-64	25 μM	6
pepstatin	1 μM	13
NEM	1 mM	0

Peptide bond specificity of MMP-PA. Peptide bonds hydrolyzed by MMP-PA in proMMP-3 and link protein (arrow)

are aligned in Table 4 to show similar or identical amino acids at five positions in the decapeptides that encompass the cleavage sites in the two substrates. Shown below for comparison are sites with arginine at P_1' in various peptides cleaved by N-arginine dibasic convertase (NRD) or insulin degrading enzyme (IDE) (compiled from Chesneau *et al.*, *J. Biol. Chem.* 269:2056-2061, 1994, and Muller *et al.*, *Biochem.* 31:11138-11143, 1992).

Table 4.

MMP-PA

DSDTLEVM	RK	proMMP-3
DNYTLDHD	RA	link

⇑

NRD

NPAMAPRE	RK	somatostatin-28
PRRPYILK	RA	preproneurotensin
YGGFLR	RI	dynorphin A
QNCPLGGK	RA	pro-ocytocin/neurophysin

IDE

GLGCNVLR	RY	brain natriuretic peptide
DSGCFG	RR	brain natriuretic peptide
FGRRLD	RI	brain natriuretic peptide
CFGLKLD	RI	C-type natriuretic peptide
FGGRID	RI	atrial natriuretic peptide

Propeptide catabolites can serve as novel indicators of progressive cartilage loss. As described above, the progressive destruction of cartilage at the articular surfaces results from increased proteolytic

activity. The novel proteinase described herein, which activates MMP-3 (stromelysin) and MMP-9 (92-k gelatinase), is produced by articular cartilage and may regulate MMP activity in the avascular tissues of the joint not only during development, but also in the processes of growth, repair, and disease. Excessive activity of MMP-PA in conditions such as osteoarthritis would disrupt the tight regulation of ECM degrading enzymes and, ultimately, cause cartilage destruction. The compositions and methods described herein can be used to "fingerprint" MMP-PA activity in biological samples, and thereby detect and diagnose conditions in which tissue is destroyed.

In addition to activating MMP-3 and MMP-9, MMP-PA may activate an entire degradative cascade in cartilage. To examine the extent of MMP-PA involvement, MMP-3, -9, -13, and "aggrecanase" proenzyme catabolites will be examined in cartilage and synovial fluids for terminal amino acid sequence consistent with *in vivo* activity of MMP-PA. These MMPs are present in adult cartilage and zymogen activation is the rate-limiting step in proteolysis catalyzed by these enzymes (*i.e.*, MMP-3, -9, -13, and "aggrecanase" proenzymes).

Catabolites generated during activation of these proenzymes are strongly correlated with progressive degenerative pathologies. Thus, by identifying specific catabolites in the synovial fluid of affected patients, one can monitor and define critical steps in disease progression and assess the effect of therapeutic agents aimed at slowing, halting, or preventing that progress.

Several MMPs, including MMP-1, MMP-3, MMP-9, and MMP-13, are up-regulated by IL-1 in cartilage. Moreover, once the zymogen forms are activated, they are capable of digesting the major matrix components (Treadwell *et al.*, *Arch. Biochem. Biophys.* 251:715-723,1986; Stephenson *et*

al., *Biochem. Biophys. Res. Commun.* 144:583-90, 1987; Mohtai *et al.*, *J. Clin. Invest.* 92:179-185, 1993; Borden *et al.*, *J. Biol. Chem.* 271:23577-81, 1996; Reboul *et al.*, *J. Clin. Invest.* 97:2011-2019, 1996). Activation of MMP zymogens involves the removal of a conserved N-terminal propeptide, most likely through proteolytic cleavage. Since these enzymes are secreted and freely diffusible through the extracellular matrix, zymogen activation at the cell surface could localize their activity. MMP-3 and MMP-9 may be especially important for cartilage degradation. Active forms of these enzymes are generated in explant cultures of osteoarthritic cartilage (Mohtai *et al.*, *J. Clin. Invest.* 92:179-185, 1993; Dean *et al.*, *J. Clin. Invest.* 84:678-685, 1989). Such explant cultures are among those that can be used to assay modulators of MMP-PA activity (*e.g.*, molecules or compounds that alter the expression or activity of MMP-PAs). In addition, degradation products consistent with cleavage at sites targeted by MMPs are present in cartilage and synovial fluid obtained from patients with osteoarthritis.

MMPs are normally tightly regulated by the requirement for zymogen activation. Despite the low turnover of normal adult articular cartilage, MMPs are expressed in both normal and osteoarthritic cartilage. Adult cartilage explants, whether normal or osteoarthritic, undergo rapid degradation in response to the MMP activator p-aminophenyl mercuric acetate. The major aggrecan catabolites result from cleavage at the MMP site (Flannery *et al.*, *J. Biol. Chem.* 267:1008-1014, 1992). Thus, a substantial pool of proMMPs exists in adult cartilage, which further suggests that zymogen activation is the rate-limiting step in degradation catalyzed by these enzymes.

Depletion of chondroitin sulfate-rich aggrecan catabolites, which is the earliest event in matrix degradation, is catalyzed not by any of the well-characterized metalloproteinases but by "aggrecanase," which cleaves aggrecan between glutamic acid and alanine in the interglobular domain and, perhaps, elsewhere (Sandy *et al.*, *J. Biol. Chem.* 266:8683-8885, 1991; Lark *et al.*, *Biochem. J.* 307:245-252, 1995; Loulakis *et al.*, *Biochem. J.* 284:589-593, 1992; Ilic *et al.*, *Arch. Biochem. Biophys.* 294:115-122, 1992). Aggrecan degrading metalloproteinase (aggrecanase) is synthesized as a precursor but may be secreted in active form. Analysis of the ADMP sequence revealed a cysteine switch-like sequence and a potential furin-cleavage site (polybasic region), suggesting a typical though perhaps intracellular mechanism of proenzyme activation.

MMPs may be involved later in the progression of cartilage degradation. For example, they may catalyze secondary cleavages of aggrecan and other non-collagenous components of cartilage and, perhaps more importantly, catalyze the destruction of collagen. Aggrecan molecules that have been lost can be newly synthesized and thereby replaced, but the destruction of the collagen framework is irreversible.

The foregoing demonstrates that distinct phases occur during the loss of articular cartilage. Aggrecanase acts earlier to release the chondroitin sulfate-rich aggrecan fragments from their hyaluronan anchor in the matrix, and the MMPs (collagenases, gelatinases, and stromelysins) are activated later to trigger the "point of no return," where excessive catabolism of structural elements overwhelms the chondrocyte's attempts to repair the damaged extracellular matrix. The rate limiting step in the damage catalyzed by these enzymes is zymogen activation.

The MMP-PA described herein directly activates the MMP-3 and MMP-9 proenzymes by cleaving at specific sites in the propeptides. Because MMP-3 can activate or contribute to the activation of several other MMPs, including MMP-1, MMP-8, and MMP-13 (collagenases) and MMP-9 (92 k gelatinase), MMP-PA has the potential to initiate a cascade of extracellular matrix degrading MMPs (such cascades are described, *e.g.*, by Knauper *et al.*, *Biochem. J.* 295:581-586, 1993; Knauper *et al.*, *J. Biol. Chem.* 271:1544-1550, 1996; Nagase *et al.*, in *Joint Cartilage Degradation: Basic and Clinical Aspects*, J.F. Woessner and D.S. Howell (Eds.), Marcel Dekker, Inc., 1993). Increased MMP-PA activity would lead to excessive catabolism of extracellular matrix under pathologic conditions, such as osteoarthritis, and plays a role at multiple steps in the progressive degeneration of osteoarthritis.

A stepwise mechanism for the activation of MMP zymogens has been described in which a proteinase initially cleaves within a proteinase-susceptible region of the propeptide (Nagase *et al.*, in *Joint Cartilage Degradation: Basic and Clinical Aspects*, J.F. Woessner and D.S. Howell (Eds.), Marcel Dekker, Inc., 1993). This initial activation product is then converted in bimolecular "autocatalytic" reaction(s) to a more stable active form having a phenylalanine or tyrosine NH_2-terminus. Similar stepwise processing through a transient 53 kDa activation intermediate occurred upon incubation of proMMP-3 with MMP-PA. In the presence of TIMP, which inhibits the autocatalytic conversion of the 53 kDa species to the 45 kDa species, a 46 kDa product accumulated to levels sufficient for N-terminal sequence analysis. A dibasic site in the proMMP-3 molecule was targeted under these conditions, with cleavage occuring on the amino side of an arginine residue. This cleavage

is secondary to a cleavage that creates a transient activation intermediate (~53 kDa), a size consistent with cleavage at another basic region closer to the amino terminus. A polybasic sequence reportedly exists in the aggrecanase propeptide (postulated furin cleavage site), and MMP-PA may target that site. Proteinase-laden membranous packets (matrix vesicles, apoptotic bodies, podosomes) are released from chondrocytes in the later degenerative phase of osteoarthritis. The precise origin of these extravasated membranes is unknown. It is possible that MMP-PA is localized intracellularly within endosomes, where it catalyzes proAggrecanase activation early in the process of articular cartilage loss, and that release of these structures as apoptotic bodies later in the degenerative process results in activation of secreted proMMPs (Hashimoto *et al.*, *Arth. and Rheum.* 41:1632-1638, 1998; Yang *et al.*, *Exp. Cell Res.* 235:370-373, 1997).

The examples below describe: (1) creation of tagged synthetic propeptide-like substrates that can be used to assay and characterize proenzyme activator(s) endogenous to osteoarthritic cartilage (including MMP-PA); (2) use these polypeptides in osteoarthritic cartilage explants and purified systems to define sites of natural and MMP-PA-catalyzed activating cleavages in the MMP-3, MMP-9, and MMP-13 propeptides (and perhaps proAggrecanase); and (3) production of neoepitope, capture, and detection reagents useful for ELISA-based measurement of proenzyme catabolites in synovial fluid. These reagents can be used not only to clarify the role of MMP-PAs in regulating enzymes that become activated in osteoarthritis and other degenerative conditions, but also to evaluate proenzyme catabolites as markers for critical steps in the progression of these conditions.

1. *Expression of recombinant MMP-3, MMP-9, MMP-13, and Aggrecanase propeptides to assay putative MMP activators and to further define pathology-related and MMP-PA-catalyzed cleavage site(s).* It is of considerable value to define the initial sites that are cleaved in the stepwise activation of zymogens in an explant culture system of osteoarthritic cartilage. It is also of value to define the site targeted by MMP-PA to generate the transient 53 kDa MMP-3 activation intermediate that is subsequently clipped to create a phenylalanine or tyrosine N-terminus and to identify cleavages in the MMP-9 and MMP-13 propeptides. To avoid secondary MMP-dependent "autocatalysis," bacteria are used to express recombinant propeptides that are truncated at the junction to the catalytic domain. These propeptide sequences have carboxy terminal hexahistidine tails to enable purification on nickel resin or adherence to metal chelate plates. An epitope tag at the N-terminus allows immunodetection of full length plate-bound propeptide but not propeptide that has been clipped. These tagged recombinant propeptides can be used to develop ELISA-based assays of clipping activity (which reflects proMMP-activating activity) in biological fluids such as cartilage extracts or explant culture media. These assays can also be used to analyze the clipping activity of purified enzymes, or to assess the biological activity of fragments thereof.

In addition, ELISA-based assays are useful to define sites of proteolysis within the propeptide in the absence of secondary bimolecular "autocatalysis," since catabolites with newly generated N-termini can be conveniently purified for sequencing. Comparison of propeptide cleavage sites in an osteoarthritic cartilage explant system with sites cleaved by MMP-PA will clarify the role of this enzyme in MMP proenzyme activation.

This information will be used to create neoepitope antisera, as described below. This approach will define sites cleaved in the natural pathology and allow the development of appropriate reagents to detect these catabolites.

2. *Preparation of antisera to the N-terminal neoepitopes generated by proteolysis of the MMP-3, MMP-9, MMP-13, and Aggrecanase propeptides.* Neoepitope technology is based on newly created epitopes (new amino and carboxy terminal ends) generated when a protein is cleaved. Antibodies raised against synthetic peptides corresponding to these epitopes are utilized in the identification or detection of these cleaved products in biological fluid. Therefore, as with protein fragment sequencing, proteinase(s) responsible for specific cleavages are identified indirectly. This method has been used to identify matrix metalloproteinase-cleaved polypeptides amongst aggrecan digestion products in cartilage tumors (Toriyama *et al.*, *Eur. J. Cancer* 34:1969, 1998).

Antisera will be prepared to detect propeptide catabolites generated by specific clipping at MMP-PA cleavage sites. Synthetic peptides corresponding to the N-terminal neoepitopes (for example KDVKQFV (SEQ ID NO:1), RRKDSGP (SEQ ID NO:2), SDTLEVM (SEQ ID NO:3), and RKPRCGV (SEQ ID NO:4) in the proMMP-3 propeptide and GYTRVAEM (SEQ ID NO:5), RGESKSL (SEQ ID NO:6), SATLKAM (SEQ ID NO:7), and RTPRCGV (SEQ ID NO:8) in the proMMP-9 propeptide) will be crosslinked to keyhole limpet hemocyanin through a C-terminal cysteine residue. The antiserum is raised by immunizing rabbits against the carrier-peptide conjugate. The protocol employs immunization with 250 μg in complete Freund's adjuvant followed by at least three boosts of the same immunogen

(100 μg) in incomplete Freund's. Rabbit bleeds are assessed by ELISA using peptides and by Western blotting of activation intermediates of the proenzymes generated in explant cultures or by MMP-PA in partial reactions. Antisera will react with the appropriate digests, but not with undigested proenzyme or enzyme activated by other agents, such as trypsin. Immunoreactive signals can be abrogated by preincubation of the antiserum with the full-length immunizing peptide but not by preincubation with truncated peptides. Initial characterization of these antisera will be followed by further ELISAs using permutations of the amino acids contained in or near the cleavage site.

In addition to the neoepitope reagents, a universal detection antiserum will be produced to specifically detect activation products in the microwell plates. The target for the detection antiserum will be a synthetic peptide based on the highly conserved cysteine switch region at the carboxy terminus of the MMP propeptides (RKPRCGVPDVC (SEQ ID NO:__). Antiserum to the KLH-linked peptide will be prepared and tested as above, except that reactivity exclusively with the terminus is not required. The antiserum is likely to recognize not only most of the MMP propeptides, but also the aggrecanase propeptide. Once characterized, the antibodies can be used to identify catabolites in biological samples (*e.g.*, cartilage and synovial fluids) from osteoarthritic patients by Western blot analysis or ELISA. Antisera production can be performed using techniques routinely employed by one of ordinary skill in the art (or performed by a commercial service), and specific antibodies can be purified by peptide affinity chromatography.

3. *Development of ELISAs for the detection of metalloproteinase propeptide catabolites.* Once characterized, the reagents described above will be used to develop an ELISA for detecting and quantitating relevant catabolites in biological samples obtained from patients with osteoarthritis. The capture antisera specific for neoepitopes (IgG fraction or affinity purified antibodies) will be bound to Nunc ELISA plates in carbonate buffer. Synovial fluid (or other biological specimens) or standards (recombinant polypeptides) will be diluted in tris-buffered saline (TBS, 0.02 M Tris HCl, 150 mM NaCl, pH 7.5). Plates will be incubated for 1 hour at 22°C to allow capture of the propeptide catabolites. Plates will be washed with TBS, and the universal detection antiserum-horse radish peroxidase conjugate will be added. After 1 hour at 22°C, plates will be washed, a chromogenic substate added, and the color detected by microplate reader.

Unlike the formidable task of purifying natural catabolites from multiple specimens of osteoarthritic cartilage or synovial fluid for protein sequencing, the indirect approach described herein will allow detection and measurement of specific catabolites of proenzyme activation, which in turn serve as markers for the progression of any condition in which articular cartilage is lost.

4. *Analysis of propeptide catabolites in cartilage and synovial fluid from osteoarthritic human joints as markers of pathogenesis.* Neoepitope technology and ELISAs can be used to determine whether proMMPs, such as proMMP-3, proMMP-9, and proMMP-13 are activated by cleavage at sites defined above in the context of progressive pathogenesis. Analysis can be performed in synovial fluid specimens obtained from patients, for

example, patients undergoing arthroscopic examination or joint replacement surgery. Samples will be centrifuged to remove blood cells, and the supernatants will be frozen in aliquots at -70°C. Fluids will be treated with hyaluronidase in the presence of a cocktail of proteinase inhibitors. Aliquots of fluid will be diluted for assay by ELISA for specific propeptide catabolites. Especially in the case of proAggrecanase, which may be activated intracellularly, alternative biololgical fluids would be considered such as extracts of cartilage (*e.g.*, articular cartilage specimens could be excised at the time of total joint replacement). Cartilage can be snap frozen and stored at -80°C. Frozen specimens are then lyophilized and extracted in 10 volumes of hypotonic extraction buffer (40 mM KCl, 20 mM Tris HCl, 0.5% Triton X-100 plus a cocktail of protease inhibitors). Extracts are then assayed by ELISA, and standard curves are prepared using the appropriate recombinant propeptides.

These studies will define proenzyme catabolites (and specifically MMP-PA-generated catabolites) useful as a markers of progressive pathogenesis (*e.g.*, progressive osteoarthritis). The values obtained for synovial fluid (and blood) can be compared against the MRI grading of the extent of disease (as well as normal controls) and against the histological-histochemical grading of the extent of the disease in small segments of tissue which will, thereby, validate the assay of MMP-PA generated catabolic products in serum or synovial fluid as a screen for conditions in which articular cartilage is lost. In addition, these catabolic products can be used to assess the extent of the disease.

What is claimed is:

1. A substantially pure polypeptide, the polypeptide being a proteinase that:

(a) activates a matrix metalloproteinase proenzyme (MMP);

(b) is produced by articular cartilage;

(c) lacks caseinase and gelatinase activity;

(d) does not degrade bovine serum albumin (BSA) or plasminogen; and

(e) is inhibited by 4-(2-aminoethyl)-benzenesulfonyl-fluoride (AEBSF) or benzamidine.

2. The polypeptide of claim 1, the polypeptide being 60-70 kDa in size.

3. The polypeptide of claim 1, the polypeptide being stable at 55°C and inactivated at 70°C.

4. The polypeptide of claim 1, wherein the MMP is MMP-3.

5. The polypeptide of claim 3, wherein the polypeptide cleaves human proMMP-3 between the $methionine_{70}$-$arginine_{71}$ and $arginine_{71}$-$lysine_{72}$ bonds.

6. The polypeptide of claim 1, wherein the MMP is MMP-9.

7. The polypeptide of claim 1, wherein the polypeptide is a human polypeptide.

8. A pharmaceutical composition comprising the substantially pure polypeptide of claim 1 and a physiologically acceptable carrier therefor.

9. A substantially pure antibody that specifically binds to the polypeptide of claim 1.

10. The antibody of claim 9, wherein the antibody is a monoclonal antibody.

11. The antibody of claim 9, wherein the antibody inhibits the activity of the polypeptide of claim 1.

12. A pharmaceutical composition comprising the antibody of claim 9 and a physiologically acceptable carrier therefor.

13. A substantially pure antibody that specifically binds to a neoepitope of a polypeptide of claim 1.

14. A substantially pure polypeptide consisting of an amino acid sequence selected from the group consisting of SEQ ID NO:1 (KDVKQFV), SEQ ID NO:2 (RRKDSGP), SEQ ID NO:3 (SDTLEVM), and SEQ ID NO:4 (RKPRCGV).

15. The polypeptide of claim 14, further comprising keyhole limpet hemocyanin.

16. A substantially pure antibody that specifically binds a polypeptide of claim 14.

17. A substantially pure polypeptide consisting of an amino acid sequence selected from the group consisting of SEQ ID NO:5 (GYTRVAEM), SEQ ID NO:6 (RGESKSL), SEQ ID NO:7 (SATLKAM), and SEQ ID NO:8 (RTPRCGV).

18. The polypeptide of claim 17, further comprising keyhole limpet hemocyanin.

19. A substantially pure antibody that specifically binds a polypeptide of claim 17.

20. A method for detecting or monitoring the progression of a condition associated with the loss of articular cartilage, the method comprising contacting a biological sample obtained from a patient with an antibody of claim 9, claim 16, or claim 19.

21. The method of claim 20, wherein the condition associated with the loss of articular cartilage is osteoarthritis.

22. The method of claim 20, wherein the biological sample is a sample of cartilage or synovial fluid.

23. The method of claim 20, wherein interaction between an antibody of claim 16, or claim 19, and a MMP catabolite in the biological sample is detected by an enzyme-linked immunosorbent assay or by Western blotting.

24. A method for detecting the presence of a MMP-proenzyme activator (MMP-PA) in a biological sample, the method comprising contacting the biological sample with an anti-MMP-PA antibody.

25. A method for inhibiting the activity of an MMP-proenzyme activator (MMP-PA), the method comprising contacting a biological sample with an anti-MMP-PA antibody or a small molecule.

26. A method for treating a patient having a condition associated with the loss of articular cartilage, the method comprising administering to the patient a compound that inhibits the activity of an MMP-proenzyme activator (MMP-PA).

27. The method of claim 26, wherein the condition associated with the loss of articular cartilage is osteoarthritis.

28. The method of claim 26, wherein the compound that inhibits the activity of MMP-PA is an antibody or small molecule that specifically binds MMP-PA.

29. A method for stimulating the loss of articular cartilage, the method comprising contacting the articular cartilage with an MMP-proenzyme activator (MMP-PA) or a biologically active fragment thereof.

30. The method of claim 29, wherein the MMP-PA is a polypeptide of claim 1 or a biologically active fragment thereof.

31. A method for identifying a compound that modulates the activity of a MMP-proenzyme activator (MMP-PA), the method comprising examining the ability of MMP-PA to activates a matrix metalloproteinase (MMP) in the presence and absence of the compound, and identifying those compounds that alter the abiltiy of MMP-PA to activate MMP.

32. The method of claim 31, wherein the MMP is MMP-3 or MMP-9.

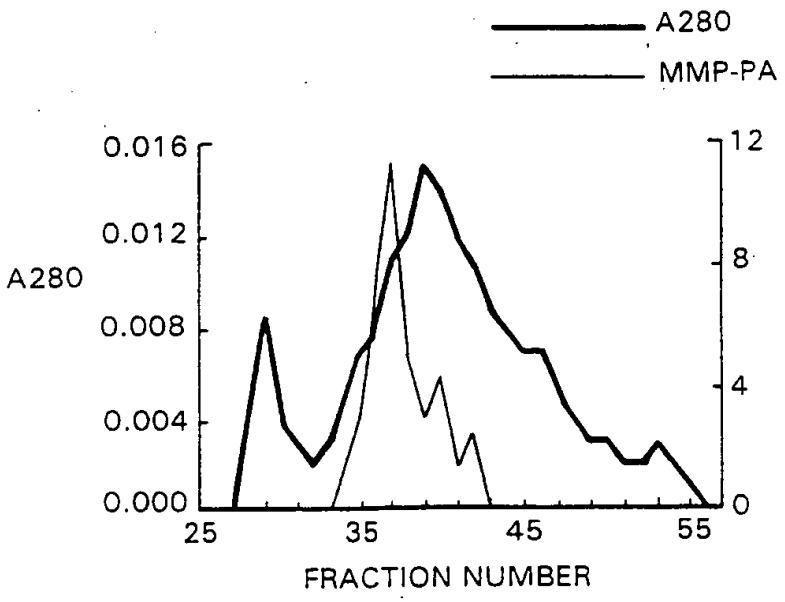
A280
MMP-PA
0.016
0.012
0.008
0.004
0.000
A280
12
8
4
0
25
35
45
55
FRACTION NUMBER

FIG. 1A

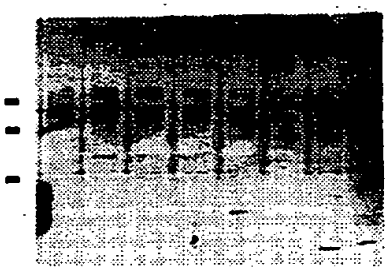

FIG. 1B

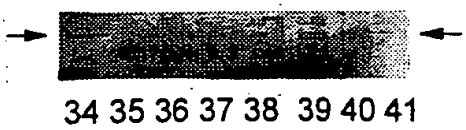

FIG. 1C

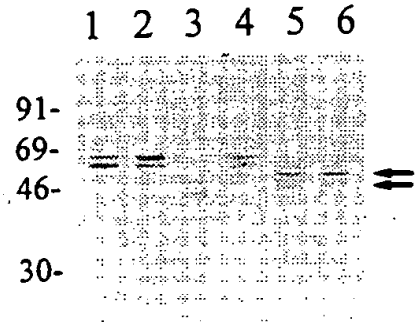

FIG. 3

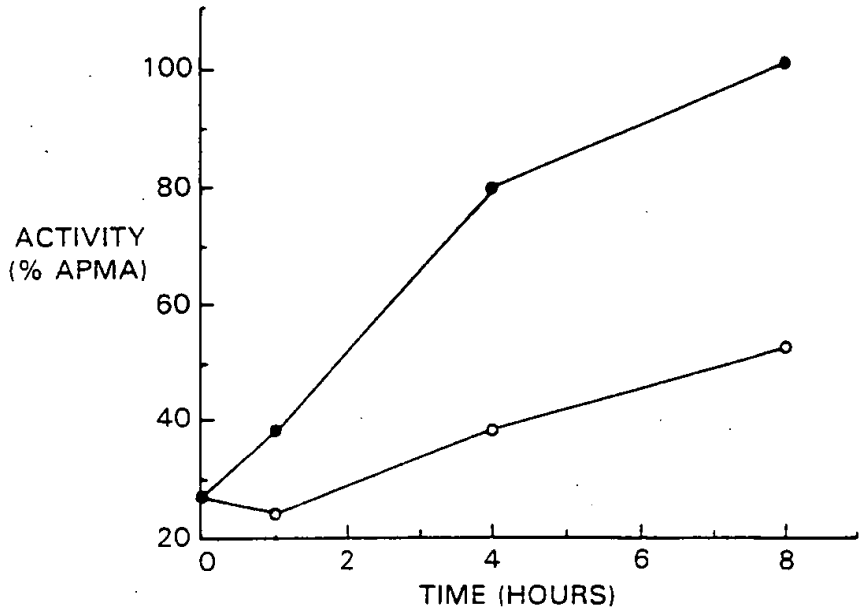
100
80
60
40
20
ACTIVITY
(% APMA)
0
2
4
6
8
TIME (HOURS)

FIG. 2A

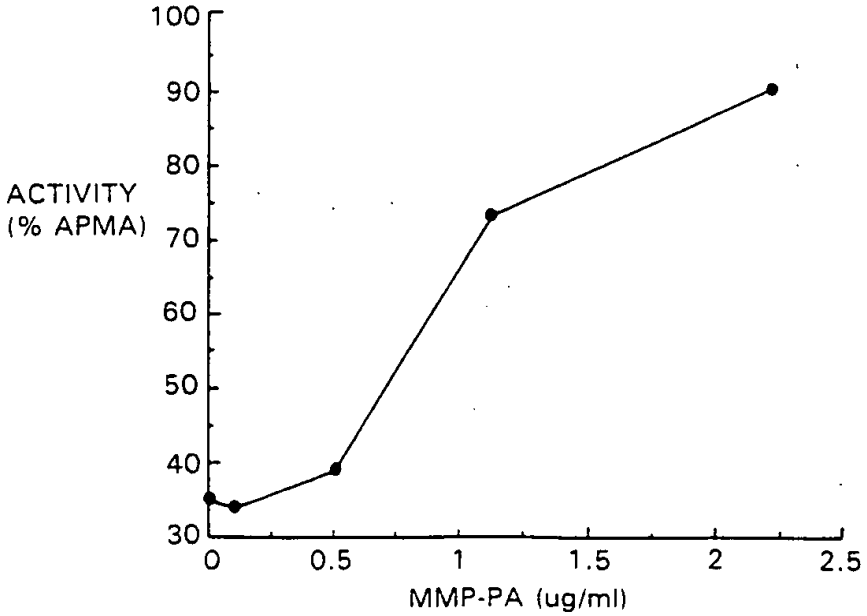
100
90
80
70
60
50
40
30
ACTIVITY
(% APMA)
0
0.5
1
1.5
2
2.5
MMP-PA (ug/ml)

FIG. 2B

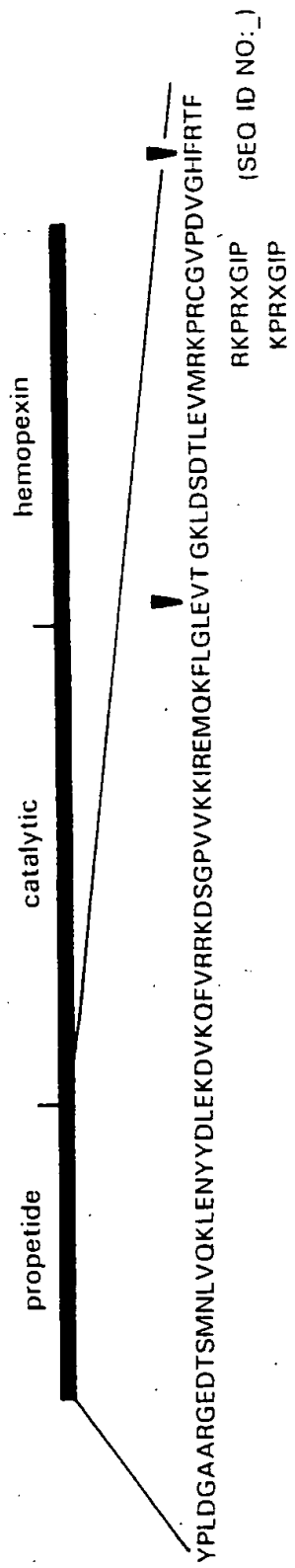
hemopexin
catalytic
propetide
YPLDGAARGEDTSMNLVQKLENYYDLEKDVKQFVRRKDSGPVVKKIREMQKFLGLEVT GKLDSDTLEVMRKPRCGVPDVGHFRTF
RKPRXGIP
KPRXGIP
(SEQ ID NO:_)

FIG. 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/13572

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 39/00, 39/395, 38/00; C07K 16/00; C12N 9/64

US CL : 424/146.1, 184.1; 435/226; 530/387.1, 323

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/146.1, 184.1; 435/226; 530/387.1, 323

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, DIALOG, EMBASE, LIFESCI, BIOSIS, CHEM ABS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FANG et al. Dog Mastocytoma Cells Secrete a 92-kD Gelatinase Activated Extracellularly by Mast Cell Chymase. J.Clin.Invest. 01 April 1996, Vol. 97, No. 7,pages 1589-1596, see entire document.	1-32
Y	US 5,585,356 A (LIOTTA ET AL) 17 December 1996(12/17/96), see entire document.	1-32

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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Date of the actual completion of the international search 29 JULY 1999	Date of mailing of the international search report 31 AUG 1999
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